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(54) Title: RECOMBINANT ANTI-CD 19 MONOCLONAL ANTIBODIES

(57) Abstract: Antibodies and methods for using them to target cells, such as abnormally proliferating B cells, are provided. In particular, chimeric, multivalent antibodies against CD 19 are provided, as well as methods of making such antibodies. The antibodies disclosed herein are useful in the treatment of diseases and disorders, particularly B cell malignancies including acute lymphoblastic leukemia, hairy cell leukemia, chronic lymphocytic leukemia, and non-Hodgkin's lymphoma.

DESCRIPTION

RECOMBINANT ANTI-CD19 MONOCLONAL ANTIBODIES

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001] The invention relates generally to the field of antibodies and methods for using them to target cells, such as abnormally proliferating B cells. More particularly, the invention concerns chimeric, multivalent antibodies against cell surface antigens, such as CD19, and methods of producing such antibodies.

2. Description of the Related Art

[0002] Some monoclonal antibodies (mAbs) can elicit anti-tumor responses by directly inhibiting growth, recruiting effector cells or molecules, or delivering cytotoxic agents to cells (Vitetta and Uhr, 1994; Carter, 2006). Some mAbs have proven useful for the treatment of human cancers (Liu *et al,* 2008). For example, anti-CD20 mAb-based therapies are used to treat B cell tumors (Haidar *et al,* 2003; Davis *et al,* 1999). However, some types of hematological tumor cells, such as pre-B acute lymphoblastic leukemia (ALL) cells do not express CD20 and are therefore resistant to anti-CD20 therapies (Davis *et al,* 1999; Kennedy *et al,* 2002). Hence, there is a need to develop novel antibodies that target alternative molecular markers expressed on some human tumors of B cell origin.

SUMMARY OF THE INVENTION

[0003] The invention regards antibodies, such as antibodies that recognize a cell surface antigen, and methods of using such antibodies. In certain aspects, chimeric, multivalent antibodies are provided. In certain embodiments, chimeric, tetravalent antibodies are provided. The antibodies may have binding specificity to a cell surface antigen. In particular embodiments, the antibodies have binding specificity to CD19. In certain embodiments, the variable regions of a rodent antibody, such as a CD19-specific antibody, are operably linked to the constant regions of a human antibody to form a chimeric antibody.

[0004] The chimeric, multivalent antibodies disclosed herein may comprise a heavy chain and a light chain. In some aspects, the heavy chain component includes a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain. In specific embodiments, the heavy chain includes all of the following components operably linked to one another in the following stated order: a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain.

[0005] The light chain component may include a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain. In specific embodiments, the light chain includes all of the following components operably linked to one another in the following stated order: a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain.

[0006] The variable domain components of antibodies disclosed herein may include one or more variable regions of the murine HD37 antibody. In some aspects, one or more of the variable regions of the murine HD37 antibody include an amino acid sequence that is 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO:13 or 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 15. In some embodiments, one or more of the variable light chain domains include an amino acid sequence that is 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 13. SEQ ID NO: 13 represents the amino acid sequence of the variable light chain domain of murine HD37 lacking the leader sequence. In some embodiments, one or more of the variable light chain domains may include an amino acid sequence that comprises SEQ ID NO: 13. In certain embodiments, one or more of the variable heavy chain domains include an amino acid sequence that is 80%>, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,

98%, 99%, or 100% identical to SEQ ID NO: 15. SEQ ID NO: 15 represents the amino acid sequence of the variable heavy chain domain of murine HD37 lacking the leader sequence. In some embodiments, one or more of the variable heavy chain domains may include an amino acid sequence that comprises SEQ ID NO: 15.

[0007] The constant domain components of antibodies disclosed herein may include one or more constant regions of the human IgGl antibody. In some embodiments, one or more of the stated constant regions of the human IgGl antibody may include an intact constant region of the human IgGl antibody or a portion of the constant region of the IgGl antibody. In some embodiments, each constant region of the human IgGl antibody comprises an intact constant region of the human IgGl antibody. The amino acid sequence of the intact constant domain of the human IgGl antibody is provided as SEQ ID NO: 17. In certain aspects, one or more of the constant regions of the human IgGl antibody include an amino acid sequence that is 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 17. In other aspects, one or more of the constant regions of the human IgGl antibody include an amino acid sequence that comprises SEQ ID NO: 17.

[0008] In some embodiments, an antibody includes a region comprising the human kappa light chain, which may include an amino acid sequence that is 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO:18. SEQ ID NO:18 represents the amino acid sequence of the human kappa light chain. In certain aspects, an antibody includes a region comprising the human kappa light chain, which may include an amino acid sequence that comprises SEQ ID NO: 18.

[0009] In some embodiments, antibodies disclosed herein may include one or more linker domains. Such linker domains may link one domain or region of the antibody to another domain or region of the antibody. For example, the heavy chain component of an antibody may include a first variable heavy chain domain linked to a second variable heavy chain domain by a linker region. In certain aspects, such a heavy chain component may include an amino acid sequence that is 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 16. In some embodiments, the

light chain component of an antibody may include a first variable light chain domain linked to a second variable light chain domain by a linker region. In certain aspects, such a light chain component may include an amino acid sequence that is 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 14.

[0010] A linker region may be any combination of three or more amino acids. In certain aspects, a linker region includes serine and glycine residues. In some embodiments, a linker region consists of only serine and glycine residues. In particular embodiments, a linker region consists of six amino acids, such as Ser-Gly-Gly-Gly-Gly-Ser.

[001 1] In certain aspects, antibodies disclosed herein include a hinge region. For example, in certain aspects, a heavy chain component of an antibody may include a first heavy chain constant domain linked to a second heavy chain constant domain by a hinge region. In some embodimetns, the hinge region may include any combination of three or more amino acids. In certain aspects, the hinge region includes 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids. In particular aspects, the hinge region includes 15 amino acids, such as the amino acid sequence provided as SEQ ID NO: 19.

[0012] In some embodiments, antibodies disclosed herein lack an Fv region that comprises tandem repeats of a single chain variable domain or a diabody construct. In certain embodiments, the antibodies lack tandem repeats of a single chain variable domain that comprise the following: VL-GGGGSGGGGSGGGGS-VH-GGGGS-VL-GGGGSGGGGSGGGGS-VH, wherein VL is the light chain variable domain, VH is the heavy chain variable domain, G is the amino acid glycine, and S is the amino acid serine. In other embodiments, the antibodies lack a diabody construct such as, for example, VH-GGGGS-VL-GGGGSGGGGSGGGGS-VH-GGGGS-VL, wherein VL is the light chain variable domain, VH is the heavy chain variable domain, G is the amino acid glycine, and S is the amino acid serine. In certain aspects, antibodies are assembled using two genes, such as a gene for the light chain component and a gene for the heavy chain component, as opposed to antibodies assembled using a single-chain gene.

[0013] The contemplated antibodies may vary in molecular mass from about 100 kDa to about 300 kDa. In some embodiments, the antibody has a molecular mass from about 150 kDa to about 250 kDa. In some aspects, the antibody may have a molecular mass of about 175 kDa to about 225 kDa. In certain aspects, a contemplated antibody has a molecular mass of about 200 kDa.

[0014] Methods for producing antibodies are also contemplated. For example, methods are provided for producing a chimeric, multivalent antibody using the following steps: (a) transfecting a vector encoding a heavy chain, light chain, or both into mammalian cells; (b) selecting the mammalian cells that express the vector of step (a); and (c) purifying the antibodies. Any suitable mammalian cell is contemplated as useful in the disclosed methods. In certain aspects, step (a) includes transfecting a vector encoding a heavy chain, light chain, or both into SP2/0, CHO/DHFR, NS0, HEK293, PerC.6, or YB2/0 cells. In some embodiments, the vector used in step (a) may encode an amino acid sequence that includes an amino acid sequence that is 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to any one of SEQ ID Nos. 13-19. In particular aspects, the vector used in step (a) encodes an amino acid sequence that comprises any one of SEQ ID Nos. 13-19.

[0015] In some embodiments, the antibodies that are purified in step (c) are antibodies that recognize a cell surface antigen. For example, the antibodies that are purified in step (c) may be anti-CD 19 antibodies. Purification of antibodies in step (c) may be by any method known to one or ordinary skill in the art. For example, purification may be precipitation, affinity purification, or both.

[0016] Also provided are pharmaceutical compositions comprising a chimeric, multivalent antibody having binding specificity to a cell surface antigen. Such pharmaceutical compositions may include any of the antibodies contemplated and disclosed herein and a pharmaceutically acceptable excipient. The phrases "pharmaceutically acceptable" or "pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable excipient" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying

agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art.

[0017] Also contemplated are uses of the antibodies and pharmaceutical compositions disclosed herein for use as a medicament. It is contemplated that any of the antibodies or pharmaceutical compositions disclosed herein may be useful as a medicament. For example, such antibodies and compositions may be used in the treatment of acute lymphoblastic leukemia, hairy cell leukemia, chronic lymphocytic leukemia, or non-Hodgkin's lymphoma and in the diseases characterized by an abnormal proliferation of B cells. In particular aspects, chimeric, multivalent antibodies that have a binding specificity to CD19 may be used in the treatment of acute lymphoblastic leukemia, hairy cell leukemia, chronic lymphocytic leukemia, or non-Hodgkin's lymphoma and in the diseases characterized by an abnormal proliferation of B cells.

[0018] A chimeric, multivalent antibody may consist of (a) a heavy chain comprising a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain; (b) a light chain comprising a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain; wherein said variable domains comprise one or more variable regions of the murine HD37 antibody and said constant domains comprise one or more constant regions of the human IgGl antibody. A linker region in such an antibody may include serine and glycine residues. In a particular embodiment, the linker region includes serine and glycine and consists of the following amino acids arranged in the stated order: Ser-Gly-Gly-Gly-Gly-Ser.

[0019] The term "antibody" encompasses proteins consisting of one or more polypeptides that are substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. Antibodies may be, for example, polyclonal, monoclonal, or mono-specific. They may be obtained by *in vitro* or *in vivo* generation of an immunogenic response. In some embodiments, antibodies are generated by recombinant DNA techniques.

[0020] Fragments of antibodies are also contemplated as useful, such as antigen-binding fragments. An "antibody fragment" is an active or functional portion of an antibody such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, and the like. Regardless of structure, an antibody fragment can bind to the same antigen that is recognized by the intact antibody. The term "antibody fragment" also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex. For example, antibody fragments include isolated fragments consisting of the light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("sFv proteins"), and minimal recognition units consisting of the amino acid residues that mimic the hypervariable region.

[0021] A chimeric (c) antibody is any recombinant protein that comprises portions from different species. For example, a chimeric antibody may contain variable domains and complementarity determining regions derived from a rodent antibody, while the remainder of the antibody molecule is derived from a human antibody.

[0022] In certain aspects, antibodies are humanized, meaning that certain non-human portions of the antibody molecule have been replaced with human portions. Such humanized antibodies may be particularly useful in therapeutic methods. Humanized antibodies may be recombinant proteins in which murine complementarity determining regions of a monoclonal antibody have been transferred from heavy and light variable chains of the murine immunoglobulin into a human variable domain.

[0023] As used herein, the term "Fc domain" is intended to refer broadly to any immunoglobulin Fc region such as an IgG, IgM, IgA, IgD or IgE Fc.

[0024] The term "vector" as used herein, refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain

vectors are capable of autonomous replication in a host cell into which they are introduced *(e.g.,* bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors *(e.g.,* non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, embodiments are intended to include such other forms of expression vectors *(e.g.,* replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

[0025] The terms "about" or "approximately" are defined as being close to as understood by one of ordinary skill in the art, and in one non-limiting embodiment the terms are defined to be within 10%, preferably within 5%, more preferably within 1%, and most preferably within 0.5%.

[0026] The term "substantially" and its variations are defined as being largely but not necessarily wholly what is specified as understood by one of ordinary skill in the art, and in one non-limiting embodiment substantially refers to ranges within 10%, within *5%,* within 1%, or within 0.5%>.

[0027] The terms "inhibiting" or "reducing" or any variation of these terms, when used in the claims and/or the specification includes any measurable decrease or complete inhibition to achieve a desired result.

[0028] The term "effective," as that term is used in the specification and/or claims, means adequate to accomplish a desired, expected, or intended result.

[0029] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

[0030] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

[0031] The compositions of the invention can comprise, consist essentially of, or consist of the claimed components. As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0032] Any embodiment of any of the present compositions and methods may consist of or consist essentially of—rather than comprise/include/contain/have—the described features and/or steps. Thus, in any of the claims, the term "consisting of or "consisting essentially of may be substituted for any of the open-ended linking verbs recited above, in order to change the scope of a given claim from what it would otherwise be using the open-ended linking verb. The phrase "consisting essentially of as used in the claims means that the claim may include additional limitations that do not materially affect the basic and novel properties of the invention as claimed.

[0033] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the examples, while indicating specific embodiments of the invention, are given by way of illustration only. Additionally, it is contemplated that changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[0034] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The

invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0035] **FIGs. 1A, IB. Construction of chimeric tetravalent and divalent cHD37 mAbs. A.** Chimeric tetravalent cHD37 mABs. **B.** Chimeric divalent cHD37 mAbs. The tetravalent cHD37 (cHD37-DcVV) was constructed by grafting additional variable domains into the divalent cHD37. A Ser-Gly-Gly-Gly-Gly-Ser linker was used to connect two VH or two VL domains, respectively. VH, heavy chain variable domain of HD37; VL, light chain variable domain of HD37; Hinge, CHI, CH2 and CH3, heavy chain constant regions of human IgGl; CL, human kappa light chain.

[0036] **FIG. 2. SDS-PAGE analysis of the purified HD37 mAbs.** A 4-15% gradient gel was performed under unreduced (lanes 2-4) and reduced (lanes 5-7) conditions. Lane 1, molecular weight markers; lanes 2 and 5, murine HD37; lanes 3 and 6, cHD37; lanes 4 and 7, cHD37-DcVV.

[0037] **FIGs. 3A, 3B. HD37 mAbs mediate ADCC against target NALM-6 or Daudi cells with murine effector LAKs. A.** ADCC against target NALM-6 cells. **B.** ADCC against target Daudi cells. *Diagonal bars* MOPC-21, *white bars* HD37, *gray bars* cHD37, *black bars* cHD37- DcVV. The background was subtracted to calculate the % specific lysis in each group. The % specific lysis of HD37 *vs.* cHD37, HD37 *vs.* cHD37-DcVV and cHD37 *vs.* cHD37-DcVV are not significantly different in either NALM-6 ($p < 0.219$) or Daudi ($p < 0.249$) cells. The figure depicts the average of three separate experiments with each carried out in triplicate ± SD.

[0038] **FIG. 4 Time-dependent ADCC against Daudi target cells with human NK effector cells at a 50:1 effector to target cell ratio.** *White bars* 6 h lysis, *black bars* 18 h lysis. The % specific lysis of HD37 *vs.* cHD37, HD37 *vs.* cHD37-DcVV, and cHD37 *vs.* cHD37-DcVV are significantly different ($p < 0.047$). Average of three separate experiments each carried out in triplicate ± SD.

[0039] **FIGs. 5A, 5B. Efficacy of HD37 mAbs in SCID mice xenografted with either NALM-6 (A) or Daudi (B) cells. A.** SCID/NALM-6 mice treated on days 1 through 4 after tumor inoculation with (·) PBS, (■) 7.5 μg/g MOPC-21, **(A)** 7.5 μg/g HD37, **(T)** 7.5 μg/g cHD37, and (♦) 10 μg/g cHD37-DcVV.

B. SCID/Daudi mice treated on days 1 through 4 post-tumor inoculation with (·) PBS, **(T)** 1.9 μg/g CHD37, **(A)** 3.8 μg/g cHD37, (■) 7.5 μg/g cHD37, **(o)** 2.5 μg/g cHD37-DcVV, (T) 5 μg/g cHD37-DcVV and (♦) 10 μg/g cHD37-DcVV. Groups of five mice were treated as described in text. The graphs are one representative of three experiments. The differences of mean paralysis times are not statistically significant between HD37 *vs.* cHD37 *vs.* cHD37-DcVV based on log-rank test.

DESCRIPTION OF THE ILLUSTRATIVE EMBODIMENTS

[0040] The inventors have designed, constructed, and expressed mouse-human chimeric, tetravalent (cHD37-DcVV) to divalent (cHD37) HD37 mAbs. The inventors also investigated the *in vitro* and *in vivo* activities of cHD37-DcVV and cHD37 mAbs against human pre-B NALM-6 cells and Burkitt's lymphoma Daudi cells. The tetravalent cHD37-DcVV mAb is expected to be superior to the divalent cHD37 for treating human tumors such as pre-B acute lymphocytic leukemias (ALLs) and non-Hodgkin's lymphomas (NHLs) due to its improved properties including enhanced binding affinity, slower dissociation rate, and improved ADCC with human effector cells.

I. CD19 and Other Cell Surface Antigens

[0041] In some embodiments, antibodies disclosed herein recognize one or more cells surface antigens and may be useful in targeting cells that express such cell surface antigens. A cell surface antigen is any antigen that is or can be expressed on the surface of a cell. In some embodiments, the cell surface antigen is a tumor-associated cell surface antigen. Specific tumor-associated antigens may be associated with a type of cancer selected from the group consisting of acute lymphoblastic leukemia, acute myelogenous leukemia, biliary, breast, cervical, chronic lymphocytic leukemia, chronic myelogenous leukemia, colorectal, endometrial, esophageal, gastric, head and neck, Hodgkin's lymphoma, lung, medullary thyroid, non-Hodgkin's lymphoma, ovarian, pancreatic, glioma, melanoma, liver cancer, prostate, and urinary bladder. A tumor-associated antigen may be selected from the group consisting of A3, the antigen specific for the A33 antibody, BrE3, CD1, CD1a, CD3, CD5, CD15, CD19, CD20, CD21, CD22, CD23, CD25, CD30, CD33, CD45, CD74, CD79a, CD80, NCA90, NCA 95, HLA-DR, CEA, CSAp, EGFR, EGP-1, EGP-2, Ep-CAM, Ba 733, HER2/neu, KC4, KS-1, KS1-4, Le-Y, S100, MAGE, MUC1, MUC2, MUC3, MUC4, PAM-4, PSA, PSMA, AFP, HCG and ist subunits, RS5, TAG-72, tenascin, IL-6, insulin growth factor-1 (IGF-1), Tn antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, VEGF, 17-1A, an angiogenesis marker, a cytokine, an immunomodulator, an oncogene marker *{e.g.,* p53), and an oncogene product.

[0042] One specific example of a cell surface antigen is CD19. CD19 is a 95 kDa transmembrane glycoprotein and member of the Ig superfamily (Tedder and

Isaacs, 1989). CD19 expression and function is confined to the B cell lineage (Chalupny *et al,* 1995; Tuveson *et al,* 1993; Uckun *et al,* 1993; Weng *et al,* 1994; Depoil *et al,* 2008; Aiba *et al,* 2008; Uckun *et al,* 1988). CD19 is expressed in pre-B ALL cells and B-ALL cells, as well as in hairy cell leukemia, chronic lymphocytic leukemia, and non-Hodgkin's lymphoma (NHL) (Uckun *et al,* 1988; Scheuermann and Racila, 1995; Anderson *et al,* 1994). Anti-CD19 mAbs can inhibit growth of tumor cells, induce cell death, recruit effector cells, reverse P-gp-mediated multidrug resistance, and deliver organic compounds and radioisotopes to target cells (Yazawa *et al,* 2005; Vallera *et al,* 2004; Zalevsky *et al,* 2009; Gerber *et al,* 2009; Bargou *et al,* 2008; Herrera *et al,* 2006; Chu *et al,* 2008; Ghetie *et al,* 1994; Ghetie *et al,* 2004). In addition, HD37 is a murine anti-human CD19 mAb conjugated to a deglycosylated ricin toxin A chain (dgRTA) and has antitumor effects against human pre-B ALL and Burkitt's lymphomas (Herrera *et al,* 2006; Stone *et al,* 1996; Messmann *et al,* 2000; Stanciu-Herrera *et al,* 2008).

[0043] One way to try and increase the anti-tumor activity of mAbs is by increasing their valencies (Ghetie *et al,* 1997; Wolff *et al,* 1993; Meng *et al,* 2004; Miller *et al,* 2003; Liu *et al,* 2006). A divalent chimeric monoclonal antibody (cmAb) has two binding sites and a single Fc; a tetravalent cmAb contains four identical functional binding sites and the same Fc (Meng *et al,* 2004; Miller *et al,* 2003; Liu *et al,* 2006). Tetravalent cmAbs typically have a slower dissociation rate from cells, as compared to divalent cmAbs, and may induce cell growth arrest and apoptosis *in vitro* (Ghetie *et al,* 1997; Miller *et al,* 2003). Murine HD37 is a potent anti-tumor antibody against many human cancers of B cell origin, and its anti-tumor activity can be improved by covalently conjugating two molecules to create a HD37 homodimer (Herrera *et al,* 2006; Ghetie *et al,* 1994; Ghetie *et al,* 2004; Stone *et al,* 1996; Messmann *et al,* 2000; Stanciu-Herrera *et al,* 2008; Ghetie *et al,* 1997). The murine HD37 homodimer (consisting of two covalently conjugated HD37 molecules) effectively induces death in tumor cell lines *in vitro*, presumably due to hyper-crosslinking of CD19 molecules (Ghetie *et al,* 1997). In contrast, divalent HD37 does not exhibit anti-tumor activity. Because HD37 is a murine mAb, it is highly immunogenic in humans and also lacks some of the biological activities of a human IgG because it has a murine Fc portion. In addition, the HD37 homodimer has two Fc

portions, a higher molecular weight (300 kDa) and shorter half-life as compared to its divalent counterpart (Ghetie *et al.*, 1997).

[0044] Currently available anti-CD19 antibodies have many disadvantages, such as a high molecular mass, high immunogenicity in human subjects, a short half-life *in vivo,* the propensity to aggregate, or suboptimal association and dissociation constants *{e.g.,* Ghetie *et al,* 1997; Meng *et al.*, 2004; Liu *et al.*, 2006; U.S. Patent App. Pub. 20070071675). Thus, there remains a need in the art for antibodies that can target CD19-expressing cells and are suitable for human therapy that lack such disadvantages. In contrast, the antibodies disclosed herein exhibit improved properties as compared to prior antibodies including enhanced binding affinity, slower dissociation rate, improved ADCC with human effector cells, decreased propensity to aggregate, decreased immunogenicity in human subjects, decreased molecular mass, and a longer half-life *in vivo.*

II. Antibodies and Antibody Compositions

[0045] Certain embodiments are directed to antibodies that can bind to a cell surface antigen, such as CD19. As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

[0046] A typical immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one light chain (typically about 25 kD or about 214 amino acids) and one heavy chain (typically about 50-70 kD or about 446 amino acids). The C-terminus of each chain defines a constant region (C) that determines the antibody's effector function *{e.g.,* complement fixation, opsonization, etc.), while the N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

[0047] Members of the immunoglobulin family all share an immunoglobulin-like domain characterized by a centrally placed disulfide bridge that stabilizes a series of antiparallel beta strands into an immunoglobulin-like fold. Members of the family *(e.g.,* MHC class I, class II molecules, antibodies and T cell receptors) can share homology with either immunoglobulin variable or constant domains.

[0048] Full-length immunoglobulin or antibody "light chains" (generally about 25 kilodaltons (Kd), about 214 amino acids) are encoded by a variable region gene at the N-terminus (generally about 110 amino acids) and a constant region gene at the COOH-terminus. Full-length immunoglobulin or antibody "heavy chains" (generally about 50 Kd, about 446 amino acids), are similarly encoded by a variable region gene (generally encoding about 116 amino acids) and one of the constant region genes (encoding about 330 amino acids). Typically, the VL will include the portion of the light chain encoded by the VL and JL (J or joining region) gene segments, and the VH will include the portion of the heavy chain encoded by the VH, and DH (D or diversity region) and JL gene segments. The Fv antibody fragment includes the variable heavy chain and variable light chain regions.

[0049] An immunoglobulin light or heavy chain variable region typically comprises three hypervariable regions, also called complementarity determining regions or CDRs, flanked by four relatively conserved framework regions or FRs. Numerous framework regions and CDRs have been described *(see, e.g.,* Kabat *et al.,* Sequences of Proteins of Immunological Interest, U.S. Government Printing Office, NIH Publication No. 91-3242 (1991)). The sequences of the framework regions of different light or heavy chains are relatively conserved. The CDR and FR polypeptide segments are designated empirically based on sequence analysis of the Fv region of preexisting antibodies or of the DNA encoding them. From alignment of antibody sequences of interest with published sequences, framework regions and CDRs can be determined for the antibody or other ligand binding moiety of interest. The combined framework regions of the constituent light and heavy chains serve to position and align the CDRs. The CDRs are primarily responsible for binding to an epitope of an antigen and are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus of the variable region chain. Framework regions are similarly numbered.

[0050] Naturally occurring (or wild-type) antibody molecules are Y-shaped molecules consisting of four polypeptide chains, two identical heavy chains and two identical light chains, that are covalently linked together by disulfide bonds. Both types of polypeptide chains have constant regions, which do not vary or vary minimally among antibodies of the same class *(i.e.,* IgA, IgM, etc.), and variable regions. The variable regions are unique to a particular antibody and comprise a recognition element for an epitope. The carboxy-terminal regions of both heavy and light chains are conserved in sequence and are called the constant regions (also known as C-domains). The amino-terminal regions (also known as V-domains) are variable in sequence and are responsible for antibody specificity. The antibody specifically recognizes and binds to an antigen mainly via six short complementarity-determining regions (CDRs) located in the V-domains.

[0051] An antibody's specificity is determined by the variable regions located in the amino terminal regions of the light and heavy chains. The variable regions of a light chain and associated heavy chain form an "antigen binding domain" that recognizes a specific epitope; an antibody thus typically has two antigen binding domains. The antigen binding domains in a wild-type antibody are directed to the same epitope of an immunogenic protein, and a typical single wild-type antibody is thus capable of binding two molecules of the immunogenic protein at the same time. Thus, a wild-type antibody is typically monospecific *(i.e.,* directed to a unique antigen) and divalent *(i.e.,* capable of binding two molecules of antigen).

[0052] A "polyclonal antibody" is generated in an immunogenic response to a protein having many epitopes. A composition of polyclonal antibodies *(e.g.,* serum) thus includes a variety of different antibodies directed to the same and to different epitopes within the protein. Methods for producing polyclonal antibodies are known in the art *(see, e.g.,* Cooper *et al.,* Section III of Chapter 11 in: Short Protocols in Molecular Biology, 2nd Ed., Ausubel *et al.*, eds., John Wiley and Sons, New York, 1992, pages 11-37 to 11-41).

[0053] A "monospecific antibody" or "antipeptide antibody" is generated in a humoral response to a short (typically, 5 to 20 amino acids) immunogenic polypeptide that corresponds to one or a few isolated epitopes of the protein from which it is derived. A plurality of antipeptide antibodies includes a variety of different

antibodies directed to a specific portion of the protein, *i.e.,* to an amino acid sequence that contains at least one, preferably only one, epitope. Methods for producing antipeptide antibodies are known in the art *(see, e.g.,* Cooper et al, Section III of Chapter 11 in: Short Protocols in Molecular Biology, 2nd Ed., Ausubel *et al.,* eds., John Wiley and Sons, New York, 1992, pages 11-42 to 11-46).

[0054] A "monoclonal antibody" is a specific antibody that recognizes a single specific epitope of an immunogenic protein. In a plurality of a monoclonal antibody, each antibody molecule is identical to the others in the plurality. In order to isolate a monoclonal antibody, a clonal cell line that expresses, displays and/or secretes a particular monoclonal antibody is first identified; this clonal cell line can be used in one method of producing the antibodies disclosed herein. Methods for the preparation of clonal cell lines and of monoclonal antibodies expressed thereby are known in the art *(see, e.g.,* Fuller et al., Section II of Chapter 11 in: Short Protocols in Molecular Biology, 2nd Ed., Ausubel *et al.,* eds., John Wiley and Sons, New York, 1992, pages 11-22 to 11-1 1-36).

[0055] In some embodiments, an antibody is a chimeric antibody. The advantages of chimeric antibodies include decreased immunogenicity and increased in vivo stability. Techniques for constructing chimeric antibodies are well-known to those of skill in the art. As an example, Leung *et al.,* Hybridoma 13:469 (1994), describe how they produced an LL2 chimera by combining DNA sequences encoding the VK and VH domains of LL2 monoclonal antibody with respective human kappa and IgGl constant region domains.

[0056] In other embodiments, an antibody is a subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons may be found, for example, in Goldenberg *et al.,* international patent publication No. WO 91/1 1465 (1991), and in Losman *et al,* Int. J. Cancer 46: 310 (1990).

[0057] In yet other embodiments, an antibody is a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted

disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green *et al.,* Nature Genet. 7:13 (1994), Lonberg *et al,* Nature 368:856 (1994), and Taylor *et al,* Int. Immun. 6:579 (1994).

III. Therapeutic Uses for CD19 Antibodies

[0058] Methods of immunotargeting cancer cells using antibodies or antibody fragments are well known in the art. For example, U.S. Patent No. 6,306,393 describes the use of anti-CD22 antibodies in the immunotherapy of B-cell malignancies, and U.S. Patent No. 6,329,503 describes immunotargeting of cells that express serpentine transmembrane antigens.

[0059] In some embodiments, CD19 antibodies are particularly useful for targeting abnormal B cells, such as B cells whose growth is not properly controlled. For example, CD19 antibodies may be particularly useful in targeting B cell malignancies, leukemias, lymphomas, and myelomas including but not limited to multiple myeloma, Burkitt's lymphoma, cutaneous B cell lymphoma, primary follicular cutaneous B cell lymphoma, B lineage acute lymphoblastic leukemia (ALL), B cell non-Hodgkin's lymphoma (NHL), B cell chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia, hairy cell leukemia (HCL), acute myelogenous leukemia, acute myelomonocytic leukemia, chronic myelogenous leukemia, lymphosarcoma cell leukemia, splenic marginal zone lymphoma, diffuse large B cell lymphoma, B cell large cell lymphoma, malignant lymphoma, prolymphocytic leukemia (PLL), lymphoplasma cytoid lymphoma, mantle cell lymphoma, mucosa-associated lymphoid tissue (MALT) lymphoma, primary thyroid lymphoma, intravascular malignant lymphomatosis, splenic lymphoma, Hodgkin's disease, and intragraft angiotropic large-cell lymphoma.

[0060] In addition, autoimmune diseases can be associated with hyperactive B cell activity that results in autoantibody production. Inhibition of the development of autoantibody-producing cells or proliferation of such cells may be therapeutically effective in decreasing the levels of autoantibodies in autoimmune diseases. Thus, CD19 antibodies may be useful in treating certain autoimmune diseases including but

not limited to systemic lupus erythematosus, Crohn's Disease, graft-verses-host disease, Graves' disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglubulinemia, primary biliary sclerosis, pernicious anemia, Waldenstrom macroglobulinemia, hyperviscosity syndrome, macroglobulinemia, cold agglutinin disease, monoclonal gammopathy of undetermined origin, anetoderma and POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, M component, skin changes), connective tissue disease, multiple sclerosis, cystic fibrosis, rheumatoid arthritis, autoimmune pulmonary inflammation, psoriasis, Guillain-Barre syndrome, autoimmune thyroiditis, insulin dependent diabetes mellitis, autoimmune inflammatory eye disease, Goodpasture's disease, Rasmussen's encephalitis, dermatitis herpetiformis, thyoma, autoimmune polyglandular syndrome type 1, primary and secondary membranous nephropathy, cancer-associated retinopathy, autoimmune hepatitis type 1, mixed cryoglobulinemia with renal involvement, cystoid macular edema, endometriosis, IgM polyneuropathy (including Hyper IgM syndrome), demyelinating diseases, angiomatosis, and monoclonal gammopathy.

[0061] CD19-targeting antibodies may also be used to treat allergic reactions and conditions such as anaphylaxis, serum sickness, drug reactions, food allergies, insect venom allergies, mastocytosis, allergic rhinitis, hypersensitivity pneumonitis, urticaria, angioedema, eczema, atopic dermatitis, allergic contact dermatitis, erythema multiforme, Stevens-Johnson syndrome, allergic conjunctivitis, atopic keratoconjunctivitis, venereal keratoconjunctivitis, giant papillary conjunctivitis, allergic gastroenteropathy, inflammatory bowel disorder (IBD), and contact allergies, such as asthma (particularly allergic asthma), or other respiratory problems. CD19 antibodies may also be useful in managing or preventing a recipient's rejection of donor tissues, organs, or stem cells.

[0062] The anti-tumor activity of a particular antibody, such as a CD19 antibody, or combination of antibodies may be evaluated *in vivo* using a suitable animal model. For example, in xenogenic lymphoma cancer models, human lymphoma cells are introduced into immune compromised animals, such as nude or SCID mice. Efficacy may be predicted using assays, which measure inhibition of tumor formation, tumor regression or metastasis, and the like.

[0063] Use of murine or other non-human monoclonal antibodies or human/mouse chimeric mAbs may induce moderate to strong immune responses in some human patients. Accordingly, preferred monoclonal antibodies for therapeutic methods for humans are human antibodies or antibodies that are at least partially humanized that bind specifically to the target antigen with high affinity while exhibiting low or no immune response in the patient. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones et al., Nature 321:522 (1986), Riechmann et al, Nature 332:323(1988), Verhoeyen et al, Science 239:1534 (1988), Carter et al, Proc. Nat'l Acad. Sci. USA 89:4285 (1992), Sandhu, Crit. Rev. Biotech. 12:437 (1992), and Singer et al, J. Immun. 150:2844 (1993).

[0064] Methods contemplated include the administration of single anti-CD19 antibodies as well as combinations, or cocktails, of different antibodies. Two or more different antibodies that bind to CD19 may, in certain aspects, provide an improved effect compared to a single antibody. Alternatively, a combination of an anti-CD19 antibody with an antibody that binds a different antigen may provide an improved effect compared to a single antibody. Such antibody cocktails may have certain advantages. For example, they may contain mAbs that exploit different effector mechanisms or combine directly cytotoxic mAbs with mAbs that rely on immune effector functionality. Such mAbs in combination may exhibit synergistic therapeutic effects. In addition, the administration of anti-CD19 mAbs may be combined with other therapeutic agents, including but not limited to various chemotherapeutic agents and immune modulators *(e.g.,* IL-2). The anti-CD19 antibodies may be administered in their "naked" or unconjugated form, or they may have therapeutic agents conjugated to them. Additionally, bispecific antibodies may be used. Such an antibody may have one antigenic binding domain specific for CD19 and the other antigenic binding domain specific for another antigen (such as, for example, CD20). Fab antibodies or fragments of such antibodies (including fragments conjugated to other protein sequences or toxins) may also be used as therapeutic agents.

IV. Pharmaceutical Compositions

[0065] Pharmaceutical compositions contemplated herein may facilitate administration of antibodies to an organism, such as an animal *(e.g.* , a mammal, bird, fish, insect, or arachnid). Mammals include bovine, canine, equine, feline, ovine, and

porcine animals, and non-human primates. In certain aspects, a pharmaceutical composition is administered to a human subject. Multiple techniques of administering or delivering a compound exist in the art including, but not limited to, oral, rectal, *(e.g.,* an enema or suppository) aerosol *(e.g.,* for nasal or pulmonary delivery), parenteral, and topical administration. In certain aspects, antibody compositions discussed herein may be particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity or lumen of an organ.

[0066] The compositions for administration will commonly comprise a solution of the antibody molecules in a pharmaceutically acceptable carrier, such as an aqueous carrier. A variety of aqueous carriers can be used, *e.g.,* buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of antibody in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

[0067] Those skilled in the art will appreciate that when the pharmaceutical compositions are administered as agents to achieve a particular desired biological result, which may include a therapeutic or protective effect(s) (including vaccination), it may be necessary to combine a composition or compound disclosed herein with a suitable pharmaceutically acceptable carrier. The choice of pharmaceutically acceptable carrier and the preparation of the composition or compound as a therapeutic or protective agent will depend on the intended use and mode of administration. Suitable formulations and methods of administration of therapeutic agents include, but are not limited to, those for oral, pulmonary, nasal, buccal, ocular, dermal, rectal, or vaginal delivery.

[0068] Depending on the mode of delivery employed, the context-dependent functional entity can be delivered in a variety of pharmaceutically acceptable forms.

For example, the context-dependent functional entity can be delivered in the form of a solid, solution, emulsion, dispersion, micelle, liposome, and the like, incorporated into a pill, capsule, tablet, suppository, aerosol, droplet, or spray. Pills, tablets, suppositories, aerosols, powders, droplets, and sprays may have complex, multilayer structures and have a large range of sizes. Aerosols, powders, droplets, and sprays may range from small (1 micron) to large (200 micron) in size.

[0069] Pharmaceutical compositions can be used in the form of a solid, a lyophilized powder, a solution, an emulsion, a dispersion, a micelle, a liposome, and the like, wherein the resulting composition contains one or more of the targetable constructs or complexes, as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. The carriers which can be used include glucose, lactose, mannose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. Examples of a stabilizing dry agent includes triulose, preferably at concentrations of 0.1% or greater *(see, e.g.,* U.S. Pat. No. 5,314,695).

[0070] Although individual needs may vary, determination of optimal ranges for effective amounts of pharmaceutical compositions is within the skill of the art. Human doses can be extrapolated from animal studies. Generally, the dosage required to provide an effective amount of a pharmaceutical composition, which can be adjusted by one skilled in the art, will vary depending on the age, health, physical condition, weight, type and extent of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy (if any) and the nature and scope of the desired effect(s).

[0071] Dosing of therapeutic compositions is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the

disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual therapeutic agents, and can generally be estimated based on EC50 found to be effective in vitro and in vivo animal models.

[0072] The range of doses (the amount of targetable construct or complex administered) is broad, since in general the efficacy of a therapeutic effect for different mammals varies widely with doses typically being 20, 30 or even 40 times smaller (per unit body weight) in man than in the rat. In general, dosage is from 0.01 g to 100 g per kg of body weight, preferably 0.01 g to 10 g/kg of body weight, 0.01 g to 1000 mg/kg of body weight, 0.01 g to 100 mg/kg of body weight, 0.01 g to 10 mg/kg of body weight, 0.01 g to 1 mg/kg of body weight, 0.01 g to 100 g/kg of body weight, 0.01 g to 10 g/kg of body weight, 0.01 g to 1 g/kg of body weight, 0.01 g to 10 g/kg of body weight, 0.01 g to 1 g/kg of body weight, 0.01 g to 0.1 g/kg of body weight, and ranges based on the boundaries of the preceding ranges of concentrations. Thus, for example, the preceding description of dosages encompasses dosages within the range of 100 to 10 g per kg of body weight, 10 g to 1000 mg/kg of body weight, 1000 mg to 100 mg, etc.

[0073] Doses may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the targetable construct or complex in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the therapeutic agent is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

[0074] The specific dose is calculated according to the approximate body weight or surface area of the patient. Other factors in determining the appropriate dosage can include the disease or condition to be treated or prevented, the severity of the disease, the route of administration, and the age, sex and medical condition of the patient. Further refinement of the calculations necessary to determine the appropriate

dosage for treatment is routinely made by those skilled in the art, especially in light of the dosage information and assays disclosed herein. The dosage can also be determined through the use of known assays for determining dosages used in conjunction with appropriate dose-response data.

[0075] An individual patient's dosage can be adjusted as the progress of the disease is monitored. Blood levels of the targetable construct or complex in a patient can be measured to see if the dosage needs to be adjusted to reach or maintain an effective concentration. Pharmacogenomics may be used to determine which targetable constructs and/or complexes, and dosages thereof, are most likely to be effective for a given individual.

[0076] A typical pharmaceutical composition for intravenous administration may be about 0.1 to 10 mg per patient per day. Dosages from 0.1 up to about 100 mg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pa. (1980).

[0077] Antibody compositions or cocktails thereof *(e.g.,* compositions that include CD19 antibodies and other agents, such as antibodies, chemicals, small molecules, etc.) can be administered for therapeutic treatments. In therapeutic applications, compositions are administered to a patient in need thereof. For example, the antibody composition may be administered to a patient that is selected for anti-CD^ therapy. For example, such a patient may have a disease characterized by abnormal proliferation of B cells, such as ALL or CLL, or any of the other conditions, diseases, or disorders disclosed herein.

V. Examples

[0078] The following examples are included to demonstrate particular embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention. However,

those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Materials and Methods

[0079] **Construction and expression of the divalent cHD37.** The heavy and light chains of cHD37 were generated using vectors containing the human IgGl and kappa constant domains, pAH4604 and pAG4622, a gift from Dr. Sherie Morrison (Coloma *et al.,* 1992). The heavy and light variable domains of HD37 were separately amplified by PCR using cDNA prepared from HD37 hybridoma cells with primers that annealed in their respective inferred leader peptides and their constant domains. After verifying the variable domain genes, primer pairs A/B and C/D were used to amplify HD37 genes for insertion into antibody expression vectors. The primers are as follows: (A) 5'-GGGTCTAGATATCCACCATGGGATGGAGCTTGATCTTTCTCTT-3' (SEQ ID NO:l); (B) 5'-GTCTAGGAATTCGCTAGCTGAGGAGACGGTGACTGAGG-3 ' (SEQ ID NO:2); (C) 5'-GGGTCTAGATATCCACCATGGAGACAGACACACTCCTGCTATGGG-3 ' (SEQ ID NO:3); and (D) 5'-GTCTAGGAATTCGTCGACTTACGTTTGATTTCCAGCTTGGTGC-3' (SEQ ID NO:4).

[0080] The heavy and light chain constructs were co-transfected into SP2/0-AG14 cells (ATCC, Manassas, VA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stable transfectants were selected using DMEM media (Sigma, St. Louis, MO) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM glutamate, and 10 mM L-histidinol (Sigma). Positive clones were screened using human Ig specific enzyme linked immunosorbent assays (ELISAs).

[0081] **Construction and expression of the tetravalent cHD37.** The tetravalent cHD37- DcVV mAb was constructed by grafting additional variable domains into the divalent cHD37 **(FIG. 1).** In this construct, the single VH gene of

the cHD37 was replaced by a gene encoding VH-SGGGGS-VH, while the VL domain was substituted by VL-SGGGGS-VL. The VH of HD37 was modified by PCR using primer pairs E/F and G/H, respectively: (E) 5'-GTCGAGAATTCCTCGAGCACCATGGGATGGTCATGTATC-3' (SEQ ID NO:5); (F) 5'-CTAGATATATTCCGGATGAGGAAACGGTGACTGAGG-3 ' (SEQ ID NO:6); (G) 5'-CTAGATATATTCCGGAGGTGGAGGTTCACAGGTTCAGCTGCAGCAGTC-3' (SEQ ID NO:7); and (H) 5'-GTCTAGGAATTCGCTAGCTGAGGAGACGGTGACTGAGG-3' (SEQ ID NO:8). The PCR products of VH were then cut by XhoI/BspEI and BspEI/Nhel, respectively, and inserted in-frame by three fragments ligation into the pIZDHL vector (Xiao-yun Liu & Ellen S. Vitetta, unpublished data) cut by Xhol/Nhel to create the pIZDHL-VVH37 plasmid. The VL gene of HD37 was modified by PCR using primers I/J and K/L pairs, respectively: (I) 5'-CTAGATATATACGCGTCACCATGGAGACAGACACACTCCTG-3' (SEQ ID NO:9); (J) 5'-CTATATATATTCCGGATTTGATTTCCAGCTTGGTGCC-3 ' (SEQ ID NO: 10); (K) 5'-CTAGATATATTCCGGAGGAGGAGGTTCAGACATTGTGCTGACCCAATC-3' (SEQ ID NO: 11); and (L) 5'-GAACCTGTGCAGCCACCGTACGTTTGATTTCCAGCTTGGTGCC-3' (SEQ ID NO: 12). The PCR products of VL were cut by MluI/BspEI and BspEI/BsiWI, respectively, and inserted in-frame into the pIZDHL-VVH37 plasmid cut by MluI/BsiWI to create the pcHD37-DcVV plasmid.

[0082] The pcHD37-DcVV plasmid was then transfected into CHO/DHFR-cells (ATCC) using Lipofectamine™ LTX (Invitrogen). Stable transfectants were selected in IMDM media (Sigma) supplemented with 10% dialyzed FBS (Invitrogen) and 200 μg/mL Zeocin (Invitrogen). Positive clones were screened using ELISAs as described above.

[0083] **Purification of recombinant cmAbs.** mAbs in the supernatant were precipitated at 4°C with ammonium sulfate at 50% saturation, dissolved in distilled water and dialyzed overnight against PBS, pH 7.5. The dialysate was affinity-purified on Protein G Sepharose (Amersham Biosciences, Piscataway, NJ), and the bound

proteins were eluted with 0.1 M Glycine-HCl-NaN3 buffer, neutralized and dialyzed overnight against PBS, pH 7.5. The dialysate was concentrated, filter-sterilized, and stored at 4°C.

[0084] **SDS-PAGE and HPLC.** The purity and molecular weights of mAbs were analyzed by 4-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) (Amersham Biosciences) using a PhastSystem (Amersham Biosciences). The gel was stained with PhastGel Blue R (Amersham Biosciences). Further the mAbs were analyzed by size-exclusion HPLC.

[0085] **Cells.** Cell lines including the CD19-expressing human Burkitt's lymphoma cell lines Daudi, Namalwa, and Raji, human pre-B ALL cell line NALM-6, and the human FcyR-expressing U937 cell line, were obtained from ATCC and maintained in culture by serial passages in RPMI-1640 media (Sigma) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

[0086] **Binding activity as determined by Flow Cytometry.** 10^6 cells (Daudi, Namalwa, Raji, NALM-6, or U937) were incubated with dilutions of mAbs (0.01-10 μg/mL) followed by FITC-labeled goat anti-human Ig (GAHIg) under saturating conditions and analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA). The percentage of positive cells was plotted against the antibody concentration.

[0087] **Radiolabeling mAbs.** mAbs were radiolabeled with $Na^{125}I$ (Amersham Biosciences) using the lodogen reagents as previously described (Liu *et al,* 2006).

[0088] **Binding of mAbs to Daudi and NALM-6 cells.** Cells were suspended in complete RPMI-1640 medium at 10^7 cells/mL and incubated with various concentrations of radiolabeled mAbs (0.25-4 μg/10^7 cells/mL) for 60 minutes on ice. The procedure used and the calculation of binding constants *(Ka)* by Scatchard plots have been described (Meng *et al.,* 2004).

[0089] **Dissociation of mAbs from Daudi and NALM-6 cells.** The cells were suspended in complete RPMI-1640 medium at 10^7 cells/mL and incubated with 5 μg/mL of the radiolabeled mAbs for 60 minutes on ice (Meng *et al.,* 2004). The

results were expressed as the percentage reduction of the bound radioactivity *versus* time.

[0090] **Internalization of mAbs by Daudi cells.** Daudi cells were incubated with ^{125}I-labeled mAbs on ice for 1 h. After washing out the excess mAbs, the cells were resuspended at 10^6/mL in complete RPMI-1640 medium and incubated at 37°C for 2 h to measure the radioactivity that was internalized, membrane bound, or released into the medium as described previously (Liu *et al,* 2006). The results were expressed as percentage of trichloroacetic acid (TCA)-precipitable radioactivity.

[0091] **[3H] Thymidine incorporation.** Cytotoxicity of mAbs against target tumor cells was studied using [3H] thymidine incorporation assay as previously described (Meng *et al,* 2004).

[0092] **ADCC.** Human NK cells were obtained from healthy donors using the RosetteSep NK cell enrichment mixture (StemCell Technologies, Vancouver, Canada) as recommended by the manufacturer. The purity of the enriched NK cell population was 60-85% (Meng *et al,* 2004). The murine effector cells were prepared as described previously (Coleman *et al,* 2006).

[0093] The cytotoxicity assay was performed as described previously (Coleman *et al,* 2006). 10^6 target cells pre-loaded with ^{51}Cr (Amersham Biosciences) were incubated with 5-10 μg mAbs at 4°C for 30 min. After washing, the target cells were mixed with either murine or human effector cells at different ratios in complete RPMI-1640 media and incubated for 6-18 h at 37°C in a C02 incubator. 2.5% Triton X-100 (Sigma-Aldrich, St Louis, MO) was used as a positive control to determine maximum ^{51}Cr release (Meng *et al,* 2004). The plates were centrifuged, and an equal aliquot of cell supernatant from each well was measured using a gamma counter (PerkinElmer, Wellesley, MA). Specific lysis was calculated using the following equation: Specific percent lysis = 100 x [(Test ^{51}Cr released - Control ^{51}Cr released) / (Maximum ^{51}Cr released - Control ^{51}Cr released)].

[0094] **Binding of human Clq as determined by ELISA.** 96 well ELISA plates were coated with 2 μg recombinant human Clq (Sigma) per well in 100 μL PBS at pH 7.4 over night at 4°C. The plates were washed and blocked with 2% bovine serum albumin (BSA)/PBS for 2 hours at room temperature. After washing,

the plates were incubated with dilutions of the HD37 constructs in 0.5% BSA/PBS for 30 minutes at 37°C (concentration ranging from 10 to 0.1 μg/well in a volume of 100 μL/weII). After washing, 50 μï of alkaline phosphatase-GAHIg (Fc specific) conjugate (Sigma) at 1:1000 dilution in 0.5%> BSA/PBS was added and the plates were developed with 50 μL substrate p-nitrophenyl phosphate solution (Sigma) for 10-20 minutes and the absorbance was measured at 405 nm using a microplate ELISA reader (Molecular Devices, Menlo Park, CA).

[0095] **Relative binding affinity of the mAbs to the human FcRn.** Recombinant human FcRn, a gift from Dr. Pamela Bjorkman (Stanford), was radioiodinated and then incubated with HD37 mAbs. The niAb-FcRn mixture was added to human IgGSepharose and further processed as described (Liu *et al.*, 2006). The affinity was expressed as a fraction relative to the binding of human IgG which was taken as 1.00.

[0096] **CDC.** mAb pre-coated ^{51}Cr-loaded Daudi or NALM-6 target cells were mixed with various dilutions of either mouse or human serum (harvested freshly from mice and healthy human donors, respectively) in complete RPMI-1640 media. Cells were then incubated for 4 h at 37°C in 5% C02. Specific lysis was calculated as described above.

[0097] **Pharmacokinetic (PK) analysis.** Female 6 to 8 weeks of age Swiss Webster mice (Taconic, Germantown, NY) were used. The mice were injected intravenously with 3-5 x 10^7 cpm/100 μL radiolabeled mAbs, and whole body radioactivity was measured every 24 hours for 1 week in an AtomLab 100 dose calibrator (Atomic Product Corp., New York, NY). The PK parameters were determined using a noncompartmental model with the PKCALC program using data collected between 24 and 168 h (Liu *et al.*, 2006).

[0098] **Therapy of SCID mice xenografted with Daudi or NALM-6 cells.** Six to nine weeks old female SCID mice (Taconic) were inoculated intravenously in the tail veins with 5 x 10^6 Daudi or NALM-6 cells in 0.1 mL sterile PBS, pH 7.4. After 24 hours, groups of 5 mice were injected intraperitoneally with the divalent or tetravalent HD37 mAb, respectively, in four equal injections on days 1 to 4 after tumor inoculation. Mice were followed daily and were sacrificed at the onset of

paralysis, a clinical symptom which accurately predicts death. The mean paralysis time (MPT) was taken as the end point (Ghetiee*t al.,* 1994).

Results

[0099] **Design, expression and purification of the divalent and tetravalent cHD37 mAbs.** Two recombinant cHD37 mAbs were constructed, one divalent and one tetravalent. Chimerization was performed to decrease immunogenicity and to optimize effector functions and PKs in humans. A tetravalent mAb was constructed to create a construct with the same valency as a homodimer but with a single human Fc portion.

[00100] The structures of the cmAbs are depicted in **FIG. 1**. Like HD37, cHD37 has two variable regions composed of variable light chain (VL) and variable heavy chain (VH) from HD37, while cHD37-DcVV has four variable regions composed of two VLs joined together by a short flexible peptide (SGGGGS) and two VHs joined together by the same linker peptide. The antibody retains the variable regions of the murine HD37 antibody but uses the human constant regions.

[00101] The cHD37 mAbs were expressed and purified to homogeneity. The purity and molecular weight were assessed by SDS-PAGE **(FIG. 2).** Under reducing conditions, murine HD37 and cHD37 yielded two protein bands with molecular masses of ~50 kDa (heavy chain) and ~25 kDa (light chain), whereas cHD37-DcVV yielded bands of ~70 kDa (heavy chain) and ~35 kDa (light chain). SDS-PAGE analysis under nonreducing conditions showed a single band of -150 kDa for murine HD37 and cHD37, and a band of -200 kDa for cHD37-DcVV. There were no differences in the purity of the two constructs, and HPLC analysis showed that there were no aggregates or fragments in the preparations (data not shown). The results of SDS-PAGE analysis suggest that the structure of cHD37 is the same as that of murine HD37, while the cHD37-DcVV contains two extra variable region domains.

[00102] **Association and dissociation of the mAbs to NALM-6 and Daudi cells.** Experiments were designed to determine whether the recombinant cmAbs were functionally similar to the parent murine HD37 mAb. First, FACS analysis was used to determine whether the recombinant cmAbs could bind to human CD19+ pre-B

ALL NALM-6 and Burkitt's lymphoma cells *in vitro*. All three HD37 mAbs bound to CD19+ cells lines comparably (data not shown), indicating that the cHD37 mAbs retained their specificity for CD19.

[00103] The cHD37-DcVV, cHD37 and murine HD37 mAbs were then iodinated and their relative association constants *(Ka)* for CD19+ cells were determined and compared using Scatchard analysis. The results are summarized in Table 1. The association of cHD37-DcVV was significantly higher ($p < 0.004$) than that of cHD37 for both NALM-6 and Daudi cells, indicating that the cHD37-DcVV mAb has a higher relative binding affinity.

Table 1. Binding affinity of HD37 mAbs for NALM-6 cells and Daudi cells[a]

mAbs	*Ka* ($x\ 10^{y}\ M^{-1}$)	
	NALM-6[b]	Daudi[c]
Murine HD37	0.210 ± 0.051	0.203 ± 0.060
cHD37	0.309 ± 0.013	0.383 ± 0.065
cHD37-DcVV	0.555 ± 0.017	0.745 ± 0.150

[a]The affinity constants (Ka) are presented. Four experiments were performed for each cell line. Data represent means ± SD. Statistical anlaysis were calculated based on a one tail paired t test.

[b]The difference between HD37 and cHD37-DcVV for NALM-6 cells is statistically significant ($p < 0.007$); the difference between either HD37 or cHD37-DcVV and cHD37 for NALM-6 cells is statistically non-significant ($p < 0.058$).

[c]The differences between HD37 mAbs for Daudi cells are statistically significant ($p < 0.004$)

[00104] The dissociation of the radiolabeled tetravalent versus divalent mAbs from NALM-6 and Daudi cells in the presence of excess non-radiolabled competitor (HD37) was compared, and the persistence of the mAbs on the cell surface was calculated from the dissociation curves and expressed as a relative half-life *(Tin)* of dissociation *(see* Table 2). The $T_{1/2}$ of dissociation of cHD37-DcVV was significantly slower than that of the murine HD37 and cHD37 mAbs for both cell lines. The results demonstrate that the strength of binding depends on the valency of the mAb constructs.

Table 2. Dissociation of HD37 mAbs from NALM-6 and Daudi cells[a]

mAbs	$Ti_{/2}$ (min)	
	NALM-6[b]	Daudi[c]
Murine HD37	33.8 ± 5.1	47.8 ± 7.4
cHD37	30.8 ± 6.5	56.3 ± 7.8
cHD37-DcVV	46.2 ± 5.8	74.7 ± 8.5

[a]The dissociation of mAbs from cells are calculated and expressed as a half-life ($Ti_{/i}$)- Three experiments were performed. Data represents means ± SD. Statistical analysis were calculated based on a one tail paired t test.

[b]The differences between cHD37-DcVV and any divalent HD37 mAbs for NALM-6 cells are statistically significant ($p < 0.05$); the difference between HD37 and cHD37 for NALM-6 cells is statistically non-significant ($p < 0.563$).

[c]The differences between cHD37-DcVV and any divalent HD37 mAbs for Daudi cells are statistically significant ($p < 0.05$); the difference between HD37 and cHD37 for Daudi cells is statistically non-significant ($p < 0.242$).

[00105] In summary, the results of these experiments demonstrate that cHD37-DcVV has four binding sites and uses more than two of them to bind antigen simultaneously, which contributes to its increased binding affinity and slower off-rate from cells.

[00106] **Binding of the mAbs to U937 cells.** Chimerization of a mAb can optimize its effector functions in humans, which is due to the presence of the Fc region of human mAbs and the ability to efficiently bind to FCYRS expressed by effector cells. Murine and human IgG bind to murine FCYRI and FCYRIII quite well, while only human IgGl, but not murine IgGl, binds to human FCYRI and FCYRIII (Hulett and Hogarth, 1994). The next set of experiments was designed to determine the ability of HD37 constructs to effectively carry out their effector functions with both mouse and human effector cells and complement.

[00107] FACS analysis was used to evaluate the relative binding affinities of the mAbs to human FCYRI+ FCYRIII+ CD19- U937 cells, a human leukemic monocytic lymphoma cell line. Binding of mAbs to both FCYRS is dependent upon the presence of an intact human Fc region. The cHD37 and cHD37-DcVV mAbs bound to U937 equally well, but the murine HD37 bound poorly to human U937 cells, likely due to the fact that mouse Fc binds poorly to human FCYRI and FCYRIII (data

not shown). These results suggest that the cmAbs can interact with effector cells bearing the human low affinity FCYRIII and murine FCYRS due to cross-reactivity (Liu *et al.,* **2006)** and thereby mediate effector functions.

[00108] ADCC. It has been reported that the efficacy of anti-CD**19** mAbs is FcyR dependent (Yazawa *et al.,* **2005).** The cHD37 and cHD37-DcVV mAbs were tested for their ability to mediate ADCC against NALM**-6** or Daudi target cells. Either murine LAK or human NK cells were used as effectors. The activity of the cHD37-DcVV was compared to those of the divalent murine and cmAbs. All HD37 mAbs were equally effective at mediating ADCC against NALM**-6** (*p* < **0.219)** and Daudi cells (*p* < **0.249)** when murine LAK effector cells were used **(FIG. 3A and 3B).** Moreover, both the chimeric mAbs mediated ADCC against Daudi target cells when human NK effector cells were used; activity was improved over time **(FIG.** 4). In comparison to cHD37, cHD37-DcVV was more effective at killing Daudi cells when human NK cells were used as effectors. As expected, murine HD37 did not mediate ADCC with human NK effector cells due to the inability of the murine Fc to bind to human FcyRs.

[00109] Binding of the mAbs to human Clq and CDC. The binding of anti-human CD**19** mAbs to the immobilized recombinant human Clq was determined by ELISA. The results presented in Table 3 demonstrate that as compared to cHD37, cHD37-DcVV binds to human complement Clq significantly better (*p* < **0.004),** suggesting that complement binding sites may be more exposed in cHD37-DcVV. The HD37 mAb did not bind to the human Clq component.

Table 3. Binding of mAbs to recombinant human Clq [a]

mAbs	Clq binding (%)
cHD37	**100**[b]
cHD37-DcVV [c]	148.6 ± 13.6
Murine HD37	7.2 ± **6.5**

[a]Average of three separate experiments, each carried out in triplicate.

[b]The binding of cHD37 was calibrated as **100%,** while the binding to the negative control (w/o antibody) was subtracted.

[c]The difference between cHD37-DcVV and cHD37 is statistically significant (*p* < **0.004).**

[001 10] The abilities of the mAbs to mediate CDC against target cells were then determined using either human or mouse sera as sources of complement. However, none of the mAbs were able to lyse either Daudi or NALM-6 target cells with human or mouse sera (data not shown), suggesting that the binding of chimeric HD37 mAbs to complement Clq does not ensure target cell lysis (Horton *et al.*, 2008). These results demonstrate that the cHD37 mAbs have a functional Fc and further indicate that the *in vitro* efficacy of the HD37 mAb constructs depends upon the murine versus human origin of the effector cells. Because cHD37-DcVV is better than the divalent cHD37 at mediating ADCC when human effector cells are used, the cHD37-DcVV mAb should be more effective than cHD37 in humans.

[001 11] **Internalization of the mAbs by Daudi cells.** Compared to their divalent counterparts, the tetravalent cmAbs usually have lower rates of internalization, but higher membrane bound fractions. That result is due, perhaps, to hyper-crosslinking of their antigens (Ghetie *et al.*, 1997; Liu *et al.*, 2006). Furthermore, decreased internalization and prolonged membrane retention of a mAb should ensure better ADCC (Liu *et al.*, 2006). The internalization of divalent and tetravalent cHD37 mAbs into Daudi cells was evaluated. At 2 h, the divalent cHD37 was well internalized into Daudi cells, while the cHD37-DcVV had a significantly lower rate of internalization ($p < 0.005$) (Table 4). In contrast, a larger proportion of cHD37-DcVV was retained on the cell membrane ($p < 0.011$), and a smaller proportion of the cHD37-DcVV was released into the medium during incubation, thereby supporting the finding that the tetravalent mAb binds to the target with a higher relative affinity.

Table 4. Uptake of HD37 constructs into Daudi cells at 2 h[a]

Parameter measured (% TCA precipitated radioactivity)	Uptake of mAbs at 2h	
	cHD37-DcVV	cHD37
Internalized[b]	9.9 ± 1.4	16.6 ± 1.5
Membrane bound[c]	64.6 ± 3.8	52.5 ± 2.7
Released into the medium[d]	25.6 ± 2.8	30.9 ± 2.5

[a]Average of three separate experiments, each carried out in triplicate.

[b]The difference between cHD37-DcVV HD37 is statistically non-significant ($p < 0.005$).

[c]The difference between cHD37-DcVV and cHD37 is statistically significant ($p < 0.01$ 1).

[d]The difference between cHD37-DcVV and cHD37 is statistically significant ($p < 0.041$).

[001 12] **Inhibition of the cell growth.** The inhibition of the growth of NALM-6 and Daudi cells by HD37 constructs was determined using [3H]thymidine incorporation and viability assays. None of the constructs had any effect on cell viability or proliferation even at concentrations of 10^{-6} M (data not shown). Therefore, the HD37 mAbs were not acting by negative cell signaling. While the homodimers did reduce cell viability, they also had two Fc portions and this may have been involved in negative signaling through FcRs on the target cells.

[001 13] **FcRn binding and pharmacokinetics.** mAbs containing an intact Fc portion can interact with FcRs, and therefore have much longer half-lives than antibody fragments lacking an Fc. The cHD37 mAbs (divalent and tetravalent) and human IgGl had indistinguishable binding affinities for the human FcRn (1.00 *vs.* 0.93 ± 0.06) ($p < 0.761$), suggesting that the half-lives of cHD37 mAbs would be similar to that of endogenous human IgGl in humans (~20 days). In contrast, murine HD37 did not bind to human FcRn (data not shown), such that it should have a very short half-life in humans like other murine antibodies. In Swiss Webster mice, the Ti/2 of the murine HD37 (302 hrs) and cHD37-DcVV (31 1 hrs) were similar, while the cHD37 showed a slight increase in the half-life (353 hrs) (Table 5). In addition, other pharmacokinetic parameters of the HD37 mAbs were comparable. These results are in accordance with previous studies indicating that the mouse FcRn binds mouse and human IgGl with comparable binding affinities (Ober *et al.,* 2001); therefore, the half-lives of the murine versus cHD37 mAbs, were similar in mice.

Table 5. Pharmacokinetcs of the HD37 mAbs in Swiss Webster mice[a]

mAb	$T_{1/2}\beta$	AUC (H x ng/mL)	FCR (day^{-1})	MRT (h)
Murine HD37	302.2 ± 32.4	40131 ± 5932	0.055 ± 0.007	436.5 +46.8
cHD37	352.5 ± 49.6	44253 ± 6288	0.047 ± 0.007	506.8 ± 71.6
cHD37-DcVV	3 11.1 ± 30.2	40557 ± 4722	0.053 ± 0.006	447.7 ± 43.6

[a]Groups of five mice were used; three experiments were performed. Values represent means ± SD. Abbreviations: $T\iota_{/2}\beta$ = half life (beta phase); AUC = area under the curve; FCR = fractional catabolic rate; MRT = mean residence time.

[001 14] **Therapeutic efficacy of the HD37 constructs in SCID mice xenografted with human tumor cell lines.** Based on the finding that HD37 mAbs exhibited ADCC activity against pre-B ALL NALM-6 cells, SCID mice xenografted with NALM-6 cells were chosen to determine the *in vivo* anti-tumor activity of HD37 mAbs against CD19+ pre-B ALLs. SCID mice were injected with 5 x 10^6 NALM-6 tumor cells and then treated with the HD37 mAbs. In brief, groups of 5 mice were injected with 5 x 10^{-11} mol/g body weight *(i.e.,* 7.5 μg/g for divalent mAbs and 10 μg/g for cHD37-DcVV). The dose selected was based on previous studies comparing murine HD37 to Rituximab™. All three mAbs significantly improved the MPT of SCID mice bearing disseminated NALM-6 tumors as compared to PBS and the MOPC-21 isotype-matched control **(FIG. 5A).** However, there was no difference in the MPT of the groups treated with murine HD37 (66 days), cHD37 (75 days) or cHD37-DcVV (63 days). This result suggests that the optimal *in vivo* antitumor activity of mAbs is not only dependent upon their valencies, but also requires effector cells with the appropriate FcRs.

[001 15] Based on the finding that *in vitro* chimeric HD37 mAbs were able to mediate ADCC against Daudi cells, the *in vivo* anti-tumor activity of the HD37 mAbs was also determined in SCID mice with Daudi cell xenografts. SCID mice were injected with 5 x 10^6 Burkitt's lymphoma Daudi cells **(FIG. 5B).** Groups of 5 mice were injected with 5, 2.5, or 1.25 x 10^{-11} mol/g body weight of cHD37 (7.5, 3.8, or 1.9 μg/g) or cHD37-DcVV (10, 5, or 2.5 μg/g). Although all treatment regimens significantly extended the MPT of SCID/Daudi mice as compared to the PBS control, there was no significant difference in the MPT of mice treated with either the cHD37 or cHD37-DcVV mAbs at equivalent doses.

Discussion

[001 16] Purified cmAbs were expressed at good yields. The cHD37-DcVV has four binding sites, more than two of which bind CD19 simultaneously. It also has higher association and slower dissociation rates from cells. The inventors as show,

based on *in vitro* data, ADCC is the major mechanism by which the cmAbs mediate their anti-tumor activity, and potency depends on the species of effector cells that mediate their effector functions. While cHD37-DcVV and cHD37 were equally effective at mediating ADCC when murine effector cells are used *in vitro,* cHD37-DcVV antibodies were better than the divalent mAbs at mediating ADCC when human effector cells were used. All three mAbs had similar half-lives in mice and were equally effective at extending the mean survival time of SCID mice xenografted with human pre-B ALL or Burkitt's lymphoma cell lines. The cHD37-DcVV antibodies exhibited a higher binding affinity for NALM-6 cells than murine HD37, but the difference in the binding affinity between cHD37-DcVV and cHD37 was not statistically significant. Dissociation of HD37 mAbs from cells was determined to estimate the strength of binding (Kyriakos *et al.,* 1992; Ong and Mattes, 1993). By comparing the dissociation rates of the divalent and tetravalent HD37 mAbs in the presence of an excess of HD37 competitor, the inventors demonstrated that cHD37-DcVV had a higher relative binding affinity for CD19; CD19 exhibited reduced dissociation from both NALM-6 and Daudi cells. In addition, the uptake of mAbs by Daudi cells showed that a larger proportion of cHD37-DcVV was retained on the membrane and less cHD37-DcVV was internalized or released into the medium as compared to cHD37, further demonstrating that cHD37-DcVV has higher functional affinity for CD19.

[001 17] The inventors also investigated ADCC using murine LAK as effector cells. All three HD37 mAbs are able to mediate ADCC with murine LAK effector cells. However, the differences in the ability of cHD37 *versus* cHD37-DcVV to mediate ADCC were only observed when human but not mouse effector cells were used. The inventors also show that cHD37-DcVV and cHD37 is able to bind the human complement component Clq. Furthermore, cHD37-DcVV exhibited a higher binding activity for human Clq, suggesting that it has more exposed complement binding sites. The affinities of the chimeric HD37 mAbs for human FcRn are similar to that of human IgG, indicating that the cHD37 mAbs should have a completely functional CH2-CH3 domain interface and their half-lives in human should be as long as that of endogenous IgG (~ 20 days). Since human IgG binds mouse FcRn, the half-lives of the murine and cHD37 mAbs were similar in Swiss Webster mice which have mouse FcRns.

[001 18] Pre-B ALLs are immature B cell tumors that are currently treated by radiation and chemotherapy with general immunosuppression as an anticipated consequence and eventually relapse following aggressive chemotherapy (Gokbuget and Hoelzer, 2009). The inventors demonstrated that all three HD37 mAbs are able to extend the mean survival time of SCID mice xenografted with human pre-B ALL NALM-6 cells, indicating that these HD37 mAbs have the potential to treat humans with pre-B ALLs. In addition, cHD37 mAbs show marked dose dependent anti-tumor activity against Daudi cells *in vivo.* The differences between the efficacy of cHD37-DcVV and cHD37 are not observed in SCID mice, since the anti-tumor activity of the anti-CD 19 mAbs depends on their ability to mediate ADCC and *in vitro* there were no observable differences between cHD37-DcVV and cHD37 when murine effector cells were used. As reported previously, the efficacy of chimeric antibodies is difficult to evaluate in mice (Loisel *et al.*, 2007) due to the differences in FcRs on human *vs.* mouse effector cells. Therefore, in the case of human antibodies, the translation of the results from preclinical studies must rely heavily on *in vitro* studies. In this regard, the tetravalent cHD37-DcVV mAb is expected to be superior to the divalent cHD37 for treating human tumors such as pre-B ALLs and NHLs due to its enhanced binding affinity, slower dissociation rate, and improved ADCC with human effector cells.

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All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of some embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

VI. References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS

1. A chimeric, multivalent antibody having binding specificity to a cell surface antigen comprising:

(a) a heavy chain comprising a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain;

(b) a light chain comprising a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain;

wherein said variable domains comprise one or more variable regions of the murine HD37 antibody, and said constant domains comprise one or more constant regions of the human IgGl antibody.

2. The antibody of claim 1, wherein said cell surface antigen is CD19.

3. The antibody of claim 1 or 2, wherein said heavy chain comprises the stated elements arranged in the order: a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain.

4. The antibody according to any one of claims 1 to 3, wherein said light chain comprises the stated elements arranged in the order: a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain.

5. The antibody according to any one of claims 1 to 4, wherein at least one of said one or more constant regions of the human IgGl antibody comprises an intact constant region of the human IgGl antibody.

6. The antibody according to any one of claims 1 to 5, wherein each of said constant regions of the human IgGl antibody comprises an intact constant region of the human IgGl antibody.

7. The antibody according to any one of claims 1 to 6, wherein one or more of said variable regions of the murine HD37 antibody comprise an amino acid sequence that is 95% identical to SEQ ID NO: 13 or 95% identical to SEQ ID NO: 15.

8. The antibody of claim 7, wherein one ore more of said variable regions of the murine HD37 antibody comprise an amino acid sequence comprising SEQ ID NO: 13 or SEQ ID NO: 15.

9. The antibody according to any one of claims 1 to 7, wherein one or more of said constant regions of the human IgGl antibody comprise an amino acid sequence that is 95% identical to SEQ ID NO: 17.

10. The antibody of claim 9, wherein one or more of said constant reqions of the human IgGl antibody comprise an amino acid sequence comprising SEQ ID NO: 17.

11. The antibody according to any one of claims 1 to 9, wherein said region comprising the human kappa light chain comprises an amino acid sequence that is 95% identical to SEQ ID NO: 18.

12. The antibody of claim 11, wherein said region comprising the human kappa light chain comprises an amino acid sequence comprising SEQ ID NO: 18.

13. The antibody according to any one of claims 1 to 12, wherein said linker region comprises serine and glycine.

14. The antibody of claim 13, wherein said linker region comprising serine and glycine consists of Ser-Gly-Gly-Gly-Gly-Ser.

15. The antibody according to any one of claims 1 to 14, wherein said hinge region comprises SEQ ID NO: 19.

16. The antibody according to any one of claims 1 to 15, wherein said multivalent antibody is a tetravalent antibody.

17. The antibody according to any one of claims 1 to 16, further defined as lacking an Fv region that comprises tandem repeats of a single chain variable domain or a diabody construct.

18. The antibody according to any one of claims 1 to 17, wherein said antibody has a molecular mass of from about 150 to about 250 kDa.

19. The antibody of claim 18, wherein said antibody has a molecular mass of about 200 kDa.

20. A method for producing the chimeric, multivalent antibody of claim 1, comprising the steps of:

(a) transfecting a vector encoding a heavy chain, light chain, or both into mammalian cells;

(b) selecting the mammalian cells that express the vector of step (a); and

(c) purifying the antibodies.

21. The method according to claim 20, wherein said mammalian cells of step (a) or (b) are SP2/0, CHO/DHFR, NS0, HEK293, PerC.6, or YB2/0 cells.

22. The method according to claim 20 or 21, wherein said vector encodes an amino acid sequence that comprises any of SEQ ID Nos. 13-19.

23. The method according to any one of claims 20 to 22, wherein said purification step (c) comprises precipitation, affinity purification, or both.

24. The method according to any one of claims 20 to 23, wherein said antibodies of step (c) are anti-CD19 antibodies.

25. A pharmaceutical composition comprising a chimeric, multivalent antibody having binding specificity to a cell surface antigen comprising:

(a) a heavy chain comprising a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain;

(b) a light chain comprising a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain;

wherein said variable domain comprise one or more variable regions of the murine HD37 antibody and said constant domains comprise one or more constant regions of the human IgGl antibody,

and a pharmaceutically acceptable excipient.

26. The pharmaceutical composition according to claim 25, wherein said heavy chain comprises the stated elements arranged in the order: a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third constant domain.

27. The pharmaceutical composition according to claim 25 or 26, wherein said light chain comprises the stated elements arranged in the order: a light chain comprising a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain.

28. The pharmaceutical composition according to any one of claims 25 to 27, wherein said linker region comprises serine and glycine.

29. The pharmaceutical composition according to claim 28, wherein said linker region comprising serine and glycine consists of Ser-Gly-Gly-Gly-Gly-Ser.

30. The pharmaceutical composition according to any one of claims 25 to 29, wherein said chimeric, multivalent antibody has binding specificity to the cell surface antigen CD19.

31. A chimeric, multivalent antibody having binding specificity to a cell surface antigen comprising:

(a) a heavy chain comprising a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain;

(b) a light chain comprising a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain;

wherein said variable domains comprise one or more variable regions of the murine HD37 antibody and said constant domains comprise one or more constant regions of the human IgGl antibody,

for use as medicament.

32. A chimeric, multivalent antibody having binding specificity to a cell surface antigen comprising:

(a) a heavy chain comprising a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain;

(b) a light chain comprising a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain;

wherein said variable domains comprise one or more variable regions of the murine HD37 antibody and said constant domains comprise one or more constant regions of the human IgGl antibody,

for use in the treatment of acute lymphoblastic leukemia, hairy cell leukemia, chronic lymphocytic leukemia, or non-Hodgkin's lymphoma and in the diseases characterized by an abnormal proliferation of B cells.

33. The chimeric, multivalent antibody according to claim 31 or 32, wherein said heavy chain comprises the stated elements arranged in the order: a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy

chain constant domain, a hinge region, a second heavy chain constant domain, and a third constant domain.

34. The chimeric, multivalent antibody according to any one of claims 31 to 33, wherein said light chain comprises the stated elements arranged in the order: a light chain comprising a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain.

35. The chimeric, multivalent antibody according to any one of claims 31 to 34, wherein said linker region comprises serine and glycine.

36. The chimeric, multivalent antibody according to claim 35, wherein said linker region comprising serine and glycine consists of Ser-Gly-Gly-Gly-Gly-Ser.

37. The chimeric, multivalent antibody according to any one of claims 31 to 36, wherein said chimeric, multivalent antibody has binding specificity to the cell surface antigen CD19.

38. A chimeric, multivalent antibody having binding specificity to a cell surface antigen consisting of:

(a) a heavy chain comprising a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain;

(b) a light chain comprising a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain;

wherein said variable domains comprise one or more variable regions of the murine HD37 antibody and said constant domains comprise one or more constant regions of the human IgGl antibody.

39. The antibody of claim 38 wherein said linker region comprises serine and glycine.

40. The chimeric, multivalent antibody according to claim 38, wherein said linker region comprising serine and glycine consists of Ser-Gly-Gly-Gly-Gly-Ser.

41. The antibody of claim 38 or 39, wherein said cell surface antigen is CD19.

42. A chimeric, multivalent antibody having binding specificity to a cell surface antigen consisting of:

(a) a heavy chain comprising a first variable heavy chain domain, a linker region, a second variable heavy chain domain, a first heavy chain constant domain, a hinge region, a second heavy chain constant domain, and a third heavy chain constant domain;

(b) a light chain comprising a first variable light chain domain, a linker region, a second variable light chain domain, and a region comprising the human kappa light chain;

wherein each said variable heavy chain domain comprises an amino acid sequence comprising SEQ ID NO: 15, each said variable light chain domain comprises an amino acid sequence comprising SEQ ID NO: 13, each said constant domain comprises an amino acid sequence comprising SEQ ID NO: 17, said hinge region comprises an amino acid sequence comprising SEQ ID NO: 19, said region comprising the human kappa light chain comprises an amino acid sequence comprising SEQ ID NO: 18, and said linker region comprises an amino acid sequence comprising Ser-Gly-Gly-Gly-Gly-Ser.

43. The antibody of claim 42, wherein said cell surface antigen is CD19.

FIG. 1A

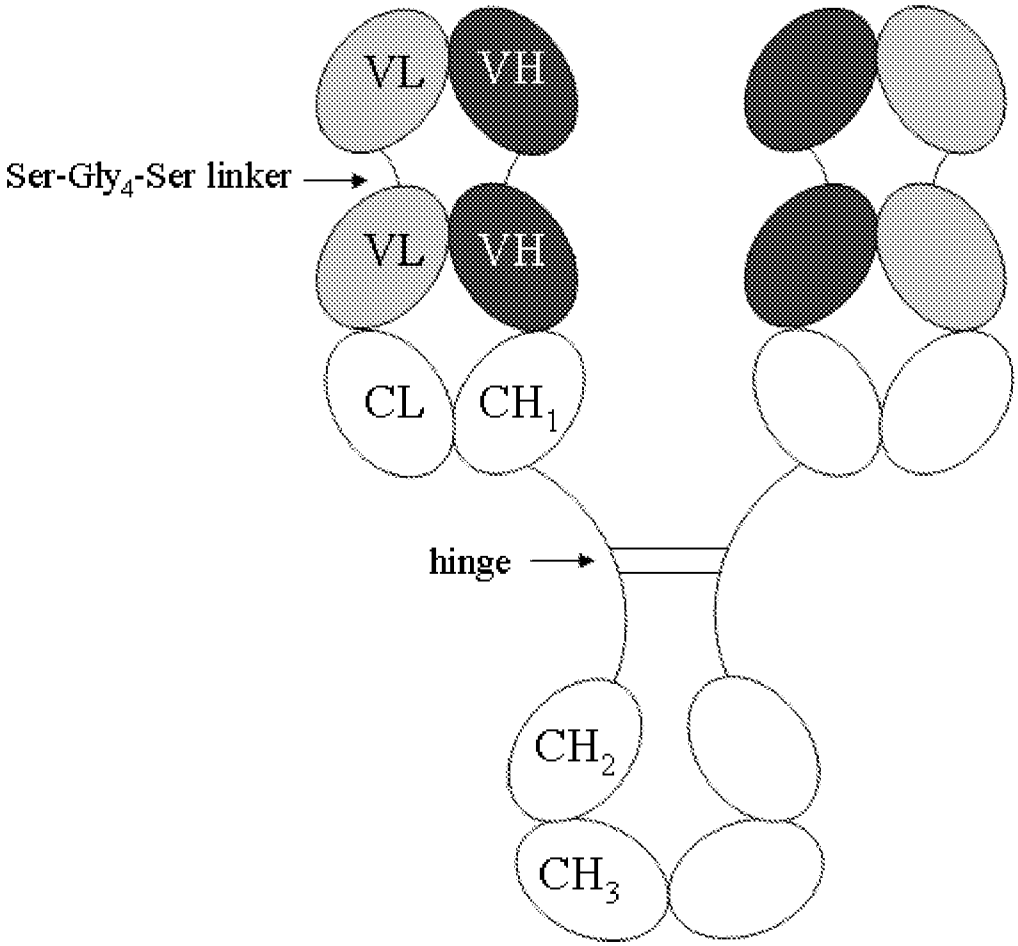


FIG. 1B

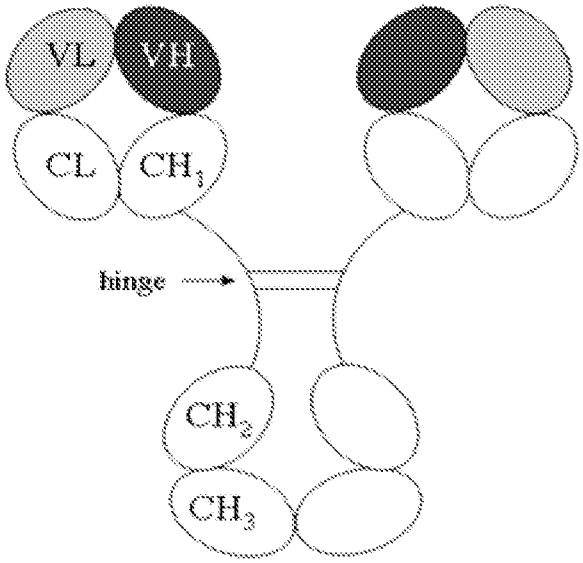


FIG. 2

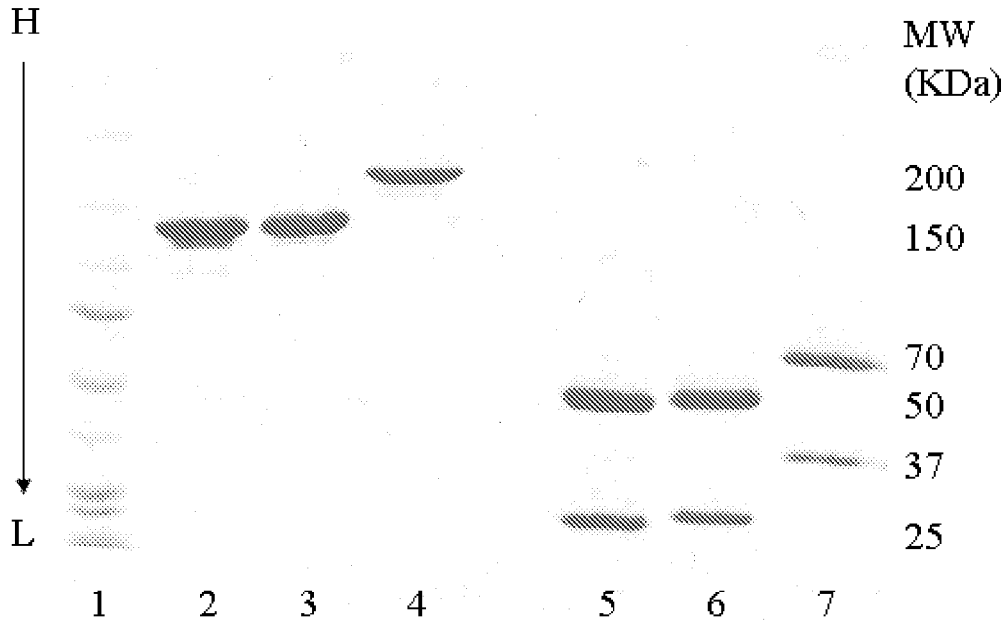


FIG. 3A

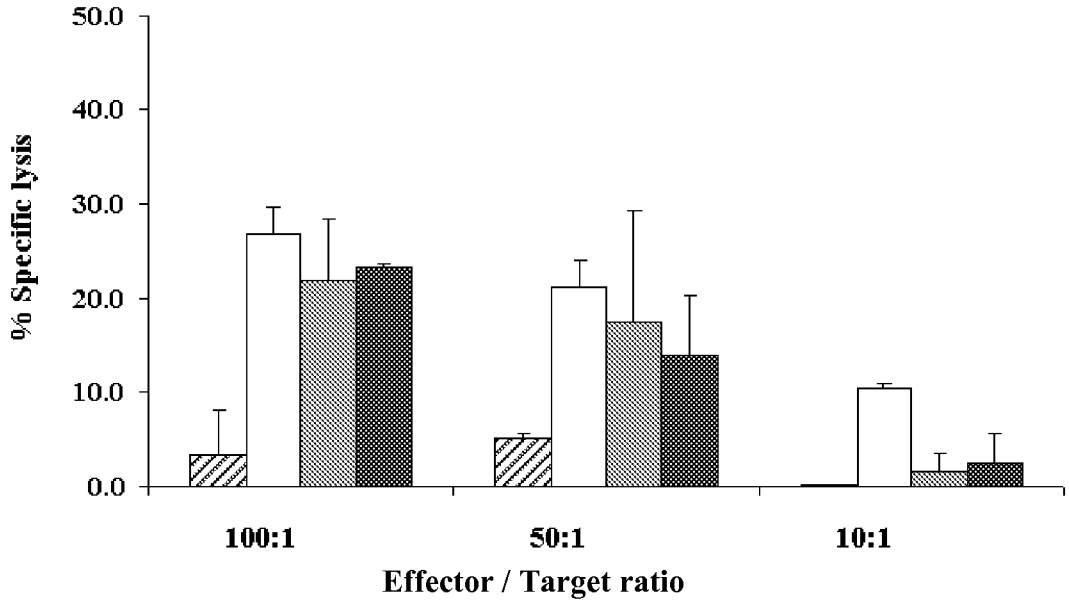
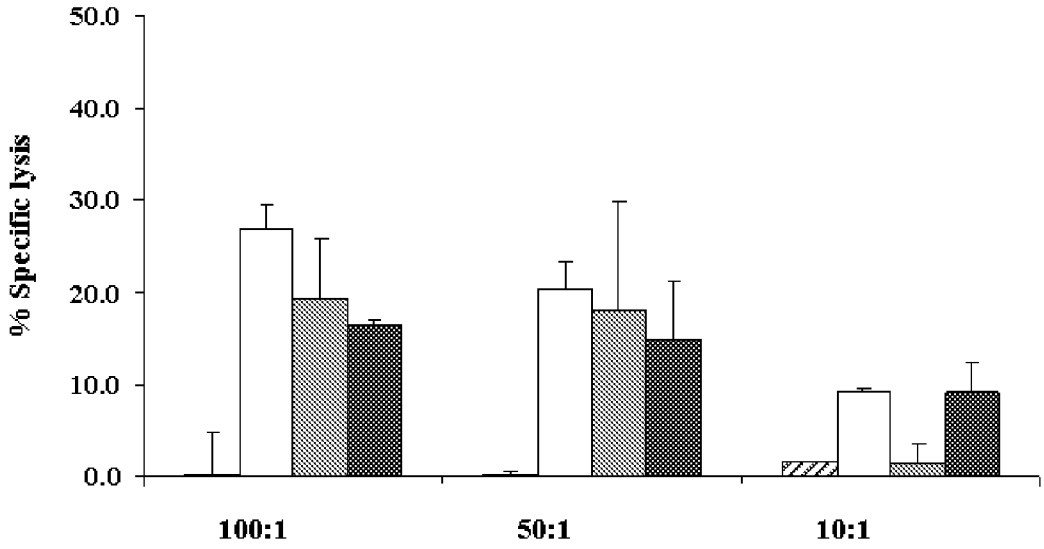

50.0
40.0
30.0
20.0
10.0
0.0
% Specific lysis
100:1
50:1
10:1
Effector / Target ratio

FIG. 3B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40.0
30.0
20.0
10.0
0.0
% Specific lysis
100:1
50:1
10:1
Effector / Target ratio

FIG. 4

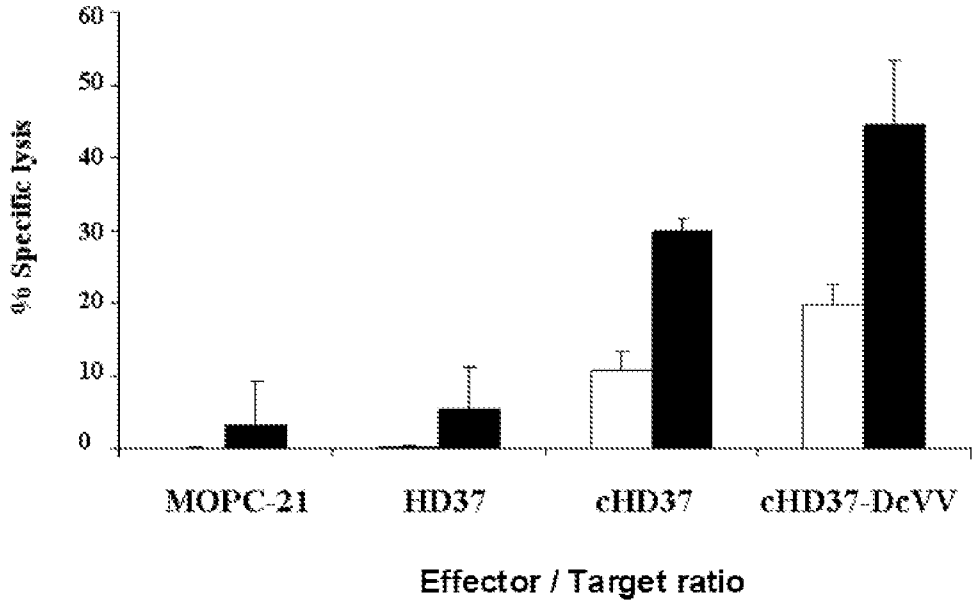

% Specific lysis
60
50
40
30
20
10
0
MOPC-21
HD37
cHD37
cHD37-DcVV
Effector / Target ratio

FIG. 5A

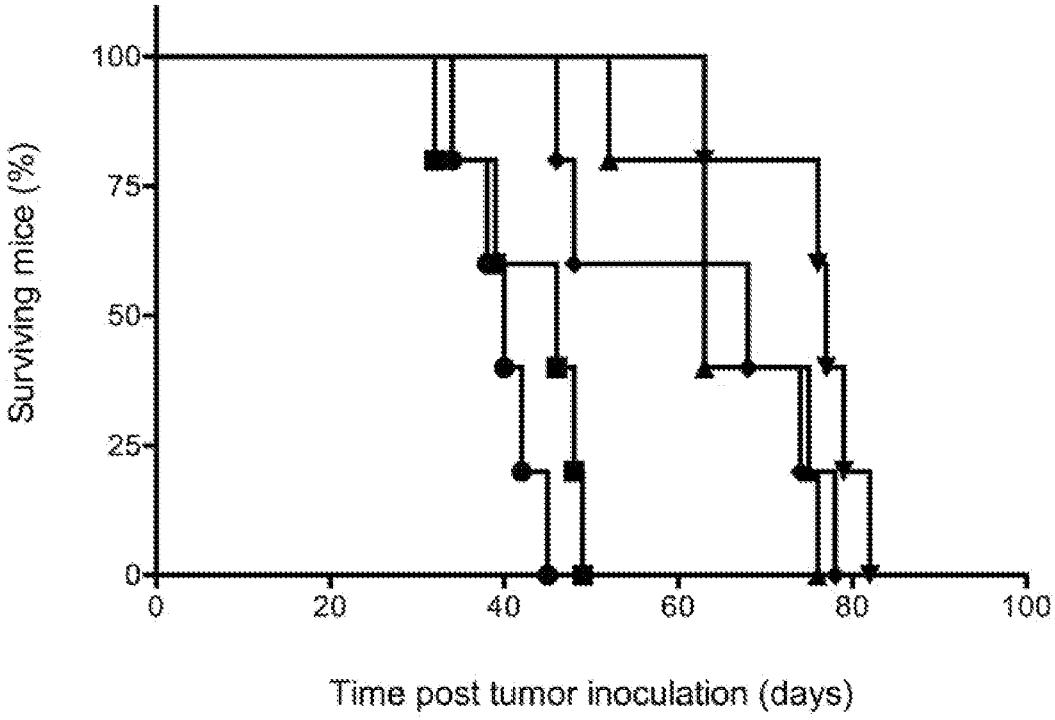
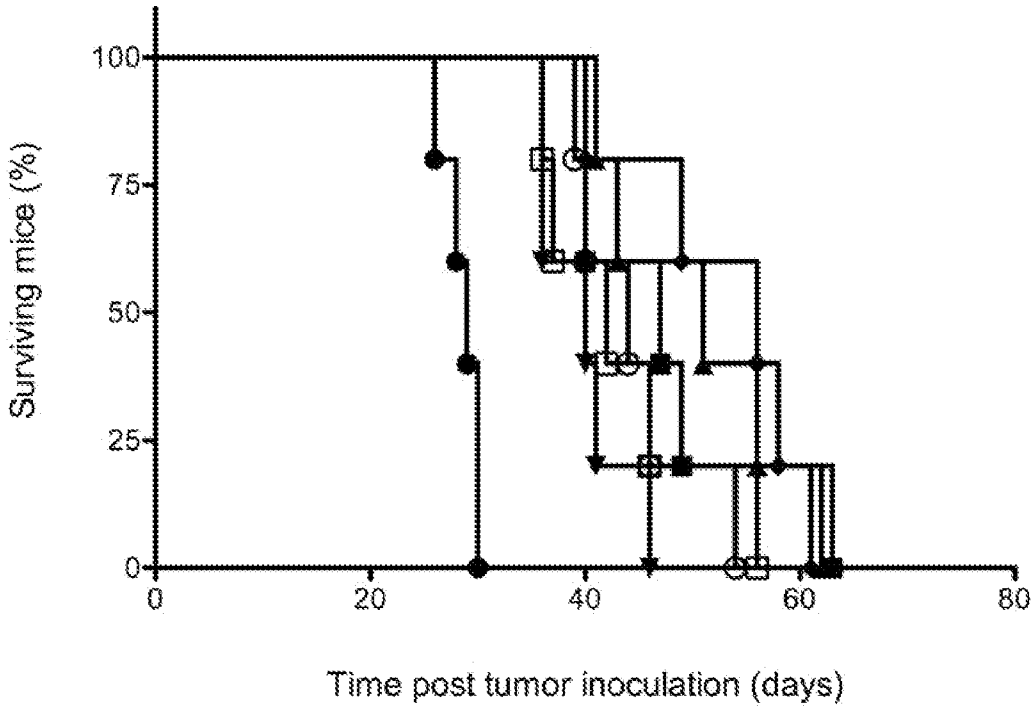

Surviving mice (%)
100
75
50
25
0
0
20
40
60
80
100
Time post tumor inoculation (days)

FIG. 5B


100
75
50
25
0
Surviving mice (%)
0
20
40
60
80
Time post tumor inoculation (days)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US201Q/05449O

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07 K16/30 A6 1K39/395 A61 P35/02
ADD .

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07 K A61 K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal , BIOSIS, CHEM ABS Data , EMBASE, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GHETIE M-A ET AL: "HOMODIMERIZATION OF TUMOR-REACTIVE MONOCLONAL ANTIBODIES MARKEDLY INCREASES THEIR ABILITY TO INDUCE GROWTH ARREST OR APOPTOSIS OF TUMOR CELLS", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, WASHINGTON, DC; US, vol . 94, 1 July 1997 (1997 -07 -01), pages 7509 - 7514, XP000941404 , ISSN: 0027 -8424 , DOI: DOI: 10.1073/ PNAS.94.14.7509 page 7512; figure 3 page 7513, paragraph 1-2; figure 6 ----- -/ -	1-43

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 2011	22/07/201 1

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Domingues , Helena

1

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/054490

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2003/165509 Al (GHETI E MARIA-ANA [US] ET AL) 4 September 2003 (2003-09-04) page 7, paragraphs 68,69 ,72 page 8, paragraph 73	1-43
Y	MENG R ET AL: "The eval uation of recombinant, chimeri c, tetravalent anti human CD22 antibodies" , CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol . 10, no. 4, 15 February 2004 (2004-02-15) , pages 1274-1281 , XP0O300552O, ISSN: 1078-0432, D0I : D0I : 10. 1158/1078-0432 . CCR-1154-03 page 1274, paragraph 2 - right-hand col umn page 1280, paragraph 1 page 1281, paragraph 1	1-43
Y	CHENGBIN W U ET AL: "Simul taneous targeting of mul t i ple di sease medi ators by a dual -variabl e-domai n immunoglobul in" , NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, NEW YORK, NY, US, vol . 25, no. 11, 1 November 2007 (2007-11-01) , pages 1290-1297 , XP002542446, ISSN: 1087-0156, D0I : D0I : 10. 1038/NBT1345 pages 1290-1 , paragraph bridgi ng; figure 1 pages 1292-3 page 1295, paragraph 2	1-43
Y	W0 2007/024715 A2 (ABBOTT LAB [US] ; WU CHENGBIN [US] ; GHAYUR TARIQ [US] ; DIXON RICHARD W) 1 March 2007 (2007-03-01) page 6, l i nes 5-14; f i gure 1 cl aims 21,22 ; examples 3.3-3 .5,4,5.2	1-43
T	XIAO-YUN LIU ET AL: "Chimeri c, di val ent and tetravalent anti -CD19 monoclonal antibodies wi t h potent i n vi tro and i n vi vo anti tumor activi t y against human B-cel l lymphoma and pre-B acute lymphoblasti c leukemi a cel l l i nes" , INTERNATIONAL JOURNAL OF CANCER, vol . 129, no. 2, 15 July 2011 (2011-07-15) , pages 497-506, XP550O2175, ISSN: 0020-7136, DOI : 10. 1002/ijc. 25695 the whole document	1-43

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US201Q/054490

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2003165509 A l	04-09-2003	US 6368596 B l	09-04-2002
W0 2007024715 A2	01-03-2007	EP 1928506 A2	11-06-2008